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ACTIVE ANTITOXIC IMMUNIZATION AGAINST RICIN USING SYNTHETIC PEPTIDES

Annual Report

Amrit K. Judd

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This year, peptides with overlapping sequences of candidate A-chain peptides, (i.e., 17-31, 84-100, 102-118, 175-191, and 227-243) were synthesized and conjugated to BSA. Immunization studies have been performed on most of the A- and B-chain peptides synthesized for the project. Antisera from the peptides were tested by ELISA for their binding to corresponding immobilized peptides, corresponding ricin subunit, and intact ricin. Challenge studies were performed on peptides eliciting cross-reacting antibodies. Although some peptides elicited high-titer, cross-reacting antibodies, none of them were able to provide protection against ricin challenge.

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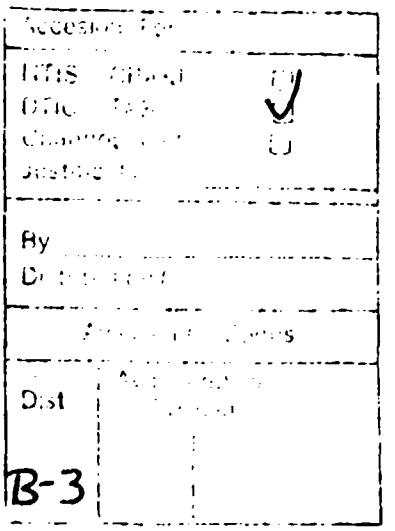
FOREWARD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council (DHHS, PHS, NIH Publication No. 86-23, Revised 1985).



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Statement of Problem

Many different plants contain cytotoxic proteins, which are among the most poisonous compounds known. The best known of these toxins is ricin, isolated from the seeds of the castor plant Ricinus communis. Because of the high toxicity of ricin, ingestion of only a few seeds can be fatal if the seed coat is broken. Extracts from castor beans have been used since ancient times for criminal purposes and biochemical warfare. No antidote for ricin poisoning is known, and no truly effective treatment exists for patients who have ingested ricin. The objective of this research is to investigate the potential of synthetic peptides derived from ricin A- and B-chain sequences to serve as immunogens for the production of protective "antitoxic" antibodies.

Background

Ricin, a plant toxin, consists of two peptide chains, A and B, linked together by a disulfide bond. The B-chain binds the toxin to receptors on the cell surface, and the A-chain enters the cytoplasm

and inactivates the 60 S ribosomal subunits, causing disruption of protein synthesis and cell death. Montfort et al. (1987) determined the three-dimensional structure of ricin and observed a reasonably prominent cleft in the A-chain enzyme assumed to be the active site.

Information regarding the immunochemistry of ricin was summarized in our proposal and is repeated below.

Sera produced against a toxoid of ricin effectively protect animals and cells in culture against intoxication with the ricin toxin. Antibodies directed against the A- and B-chains have been found to be equally efficient in preventing the effect of the toxin on animals and on cells in culture. It has also been demonstrated that anti-B-chain antibodies efficiently inhibit the binding of the B-chain to cell surfaces and that anti-A-chain antibodies prevent the enzymatic activity of the A-chain on ribosomes in a cell-free system. In experiments performed by Godab et al. (1983), and Fodstad et al. (1984), the presence of circulating immune complex was clearly demonstrated in mice, but no indications of side effects attributable to immune complexes were observed in mice or humans (cancer patients). Also, no significant level of specific IgE was found in patients' sera, suggesting that the probability of anaphylactic reactions is minimal.

So far, no work on determining the antigenic sites and mapping the epitopes on ricin chains has appeared in print.

SCIENTIFIC PROGRESS DURING THE YEAR

Peptide Synthesis

During the year we synthesized peptides with overlapping sequences of candidate A-chain peptides. The candidate peptides elicited high-titer, cross-reacting antibodies. The sequences of these peptides are shown in Table 1. Peptides were purified and then conjugated to bovine serum albumin (BSA).

Evaluation of Peptides

During the year we completed the assays on all the A- and B-chain peptides as well as peptides from A-chain with overlapping sequences. Sera to these peptides were raised in mice and tested for anti-peptide antibodies and for corresponding anti-ricin-subunit antibodies. We also performed challenge studies on ricin A-chain peptides that elicited cross-reacting antibodies.

Table 1

AMINO ACID SEQUENCES OF THE OVERLAPPING PEPTIDES FROM RICIN A-CHAIN

Peptide	Sequence
Candidate peptide	^{17}T V Q S Y T N F I R A V R G ^{31}R
Overlapping peptide	^{13}T A G A T V Q S Y T N F I R ^{27}A
Overlapping peptide	^{21}Y T N F I R A V R G R L T T ^{35}G
Candidate peptide	^{84}Y R A G N S A Y F F H P D N Q E ^{100}D
Overlapping peptide	^{80}Y V V G Y R A G N S A Y F F H P ^{96}D
Overlapping peptide	^{88}N S A Y F F H P D N Q E D A E A ^{104}I
Candidate peptide	^{102}E A I T H L F T D V Q N R Y T F ^{118}A
Overlapping peptide	^{98}Q E D A E A I T H L F T D V Q N ^{114}R
Overlapping peptide	^{106}H L F T D V Q N R Y T F A F G G ^{122}N
Candidate peptide	^{175}I S E A A R F Q Y I E G E M R T ^{191}R
Overlapping peptide	^{170}I C I Q M I S E A A ^{180}R
Overlapping peptide	^{178}A A R F Q Y I E G E ^{188}M
Overlapping peptide	^{186}G E M R T R I R Y N ^{196}R
Candidate peptide	^{227}A S P I Q L Q R D G S K E S V Y ^{243}D
Overlapping peptide	^{221}S N Q G A F A S P I Q ^{232}L
Overlapping peptide	^{230}I Q L Q R D G S K F ^{240}S
Overlapping peptide	^{238}K F S V Y D V S I L L ^{249}P

EXPERIMENTAL METHODS

Peptide Synthesis

The peptides were synthesized by solid-phase technique on a Beckman 990C Automated Peptide Synthesizer or a multiple peptide synthesizer fabricated at SRI; starting with commercially available t-Boc amino acid polystyrene resin and t-Boc protected amino acids with the following side-chain protecting groups: O-benzyl esters for Asp and Glu, O-benzyl ethers for Thr and Ser, tosyl for Arg and His, p-methoxybenzyl for Cys, orthochlorobenzyloxycarbonyl for Lys, and 2,6-dichlorobenzyl for Tyr. All couplings were performed using 2.5-molar excess of t-Boc amino acid and dicyclohexylcarbodiimide (DCC) over the number of milliequivalents of amino acid on the resin. In the case of Asn and Gln, a 2.5-molar excess of N-hydroxybenzotriazole (HOBT) was added; furthermore, if the peptides had His in their sequence then, for Asn and Gln, active ester (p-nitrophenyl) couplings were performed. All the couplings were monitored by the ninhydrin test. Couplings were repeated whenever required. For Boc deprotection, 40% TFA-CH_2Cl_2 containing 10% anisole and 0.1% indole was used. Details of the synthetic cycle are given in Table 2.

Table 2

SCHEDULE FOR PEPTIDE ASSEMBLY ON RESIN

Step	Reagent or Solvent	Time (min)
1	CH_2Cl_2 × 3	1.5
2	40% TFA/CH_2Cl_2 prewash	5
3	40% TFA/CH_2Cl_2	30
4	CH_2Cl_2 × 6	1.5
5	80% Isopropanol/CH_2Cl_2 × 3	1.5
6	CH_2Cl_2 × 3	1.5
7	5% Diisopropylethylamine/CH_2Cl_2 × 2	10
8	CH_2Cl_2 × 3	1.5
9	Coupling; 3-fold excess of t-Boc amino acid in CH_2Cl_2:DMF (9:1, v/v); DCC/CH_2Cl_2	120
10	CH_2Cl_2 × 3	1.5
11	80% Isopropanol/CH_2Cl_2 × 3	1.5

After synthesis, the peptides were cleaved from the resin using anhydrous HF in HF-Reaction Apparatus Type II (Peninsula Labs). HF (10 ml per gram of resin) was distilled into a reaction vessel containing peptide resin plus anisole (1 ml per gram of resin) and the mixture was stirred at 0°C for 45 min. HF was then evaporated completely under vacuum (first at 0°C and then at room temperature). The peptides were separated from the various organic side products by extraction with ether and isolated from the resin by extraction with 50% acetic acid, diluted, and lyophilized. The crude peptides

were purified on high-pressure liquid chromatography (HPLC) using a 20-mm x 50-cm column packed with Vydac 15- to 20-μ C_{18}.

The final peptides were obtained in 20% to 40% yield in >95% purity by HPLC. Amino acid analysis of all peptides agreed within ±10% of the theoretical values (Table 3).

For immunization, peptides were conjugated to BSA. For conjugation to BSA, BSA was first succinylated (Atassi et al., 1981). BSA (100 mg) was dissolved in 0.025 M borate buffer, pH 9.3 (20 ml). To this, a solution of succinic anhydride (540 mg) in dioxane (10 ml) was added in small aliquots over a period of 30 min and the reaction mixture was stirred magnetically while maintaining the pH at 9.3 by the addition of 3 M NaOH. Following the last addition of succinic anhydride, the acylation reaction was allowed to continue for 1 hr. The solution was then dialyzed against several changes of 0.01 M triethylamine, freeze-dried, and finally dried in a desiccator over P_2O_5. The completion of succinylation was checked with trinitrobenzene sulfonic acid.

For each peptide, 14 mg of succinyl-BSA was suspended in 1 ml of anhydrous DMF and stirred magnetically for 4 hr while shielded from direct light. p-Nitrophenol (6.5 mg) was added. The mixture was stirred magnetically for 15 min, after which 9 mg of DCC was added. The reaction mixture was allowed to stir at room temperature for 3 hr. To this was added 10 mg of the peptide to be conjugated

Table 3

AMINO ACID COMPOSITIONS OF SYNTHETIC PEPTIDES FROM A-CHAIN WITH OVERLAPPING SEQUENCES

Amino Acid	Level[a] (mol/mol) in peptide											
	13-27	21-35	80-96	88-104	98-114	106-122	170-180	178-188	186-196	221-232	230-240	238-249
Ala	3.0 (3)	1.0 (1)	1.9 (2)	2.9 (3)	2.0 (2)	1.0 (1)	2.0 (2)	2.0 (2)		2.0 (2)		
Arg	1.0 (1)	3.1 (3)	1.1 (1)		1.1 (1)	1.0 (1)	1.1 (1)	1.1 (1)	4.1 (4)		1.1 (1)	
Asn/Asp	1.0 (1)	1.0 (1)	2.1 (2)	4.0 (4)	3.0 (3)	3.0 (3)			1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)
Cys[b]							0.9 (1)					
Gln/Glu	1.0 (1)			3.0 (3)	4.0 (4)	0.9 (1)	2.0 (2)	3.0 (3)	1.0 (1)	2.1 (2)	2.0 (2)	
Gly	1.0 (1)	2.0 (2)	2.0 (2)			2.1 (2)		1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	
His			1.1 (1)	1.0 (1)	1.0 (1)	0.9 (1)						
Ile	1.0 (1)	1.0 (1)		1.0 (1)	1.0 (1)		2.9 (3)	1.0 (1)	1.0 (1)	1.0 (1)	0.9 (1)	1.0 (1)
Leu		1.0 (1)			1.0 (1)	0.9 (1)				1.1 (1)	1.0 (1)	2.1 (2)
Lys											1.0 (1)	1.0 (1)
Met							0.7 (1)	0.8 (1)	0.7 (1)			
Phe	1.0 (1)	1.0 (1)	2.1 (2)	2.0 (2)	1.0 (1)	3.1 (3)		1.0 (1)		1.0 (1)	1.0 (1)	1.0 (1)
Pro			1.0 (1)	1.0 (1)						1.0 (1)		1.1 (1)
Ser	1.0 (1)		1.0 (1)	0.9 (1)			1.0 (1)			1.8 (2)	1.9 (2)	2.0 (2)
Thr	3.0 (3)	3.0 (3)			2.0 (2)	2.0 (2)			1.0 (1)			
Tyr	1.0 (1)	1.0 (1)	3.0 (3)	1.0 (1)		1.0 (1)		1.0 (1)	1.0 (1)			0.8 (1)
Val	1.0 (1)	1.0 (1)	1.7 (2)		1.0 (1)	1.0 (1)						2.0 (2)

[a]Values in parenthesis indicate the expected number of amino acids in synthetic peptides. Values for Ser and Thr have been corrected for 10% or 15% destruction during 24 hours hydrolysis. Quantities <0.1 mol/mol have been omitted.

[b]Was determined as cysteic acid.

and 100 µL of distilled triethylamine. The reaction mixture was stirred overnight while protected from direct light, then diluted with 3 ml of water, dialyzed extensively against distilled water, and lyophilized.

The extent of conjugation, calculated from the increase in weight of the conjugate, was from 20 to 35 molecules of peptide per molecule of BSA.

Production of Anti-ricin Antisera

First, ricin toxoid was prepared by inactivating ricin with 10% formalin. After extensive dialysis, mice were immunized with this toxoid. These mice produced antibodies that bound to ricin A-chain, ricin B-chain, and ricin. Mice were also immunized with ricin A-chain, and these mice produced antibodies that bound to both ricin A-chain and ricin, as determined by immunodiffusion. Similarly, mice immunized with ricin B-chain produced antibodies that bound to both ricin B-chain and ricin.

Production of Anti-peptide Antisera

Mice were immunized with 10 or 100 µg of each immunogen (peptide, peptide-carrier conjugate, ricin subunits, or ricin) emulsified in complete Freund's adjuvant. Following primary

immunization, mice were given one to three booster immunizations, as appropriate, to elicit high-titer antiserum. Control sera and sera from the immunized mice were tested in a solid-phase immunoassay (ELISA) to determine binding activity to the immunizing peptide, the corresponding ricin subunit, and intact ricin.

Determination of LD_{100} of Ricin

We conducted five experiments to determine LD_{100} of ricin. In two experiments untreated mice were injected with various doses of ricin intraperitoneally (ip) and in three experiments intravenously (iv). From the number of surviving mice in each group, the LD_{100} was calculated to be 350 ng when injected ip and 200 ng when injected iv.

Protection Studies

In these experiments mice were immunized with peptide in complete Freund's adjuvant (CFA) on Day 0, then boosted with peptide in incomplete FReund's adjuvant (IFA) on Days 21 and 35. Control animals were consistently boosted with PBS in IFA at the time the other animals in the experiment were receiving booster injections of peptide in IFA. Mice were then challenged intravenously with 200 ng of ricin and the survival of the mice was monitored.

RESULTS AND DISCUSSION

During the year we performed binding experiments with more of the A- and B-chain peptides. Results of these experiments are discussed below and summarized in Tables 4 and 5.

We performed ELISA assays for the binding of anti-ricin A peptides to immobilized peptides, ricin A, and intact ricin. As shown in Table 4, sera from ricin A peptides 17-31 and 32-49, free as well as their BSA conjugates, exhibited significant serum antibody titer against peptide, ricin A, and intact ricin. Among the B-chain peptides, most of the BSA conjugates exhibited significant serum antibody titer against peptides but none of them had any titer against ricin B-chain or intact ricin.

During the year we performed challenge studies repetitively on all the peptides from A chain that exhibited significant serum antibody titer against ricin A-chain and intact ricin. Initially, mice immunized with ricin A peptides 175-191 and 227-243 were challenged with 300 ng of ricin (which produced 100% mortality) injected intraperitoneally. Survival rates with these peptides were found to be 100% and 90%, respectively. In a second challenge experiment, mice immunized with peptides 17-31, 17-31-BSA,

Table 4

BINDING OF SERUM FROM MICE IMMUNIZED
WITH RICIN A PEPTIDES TO THE IMMUNOGEN, RICIN A, AND RICIN

Ricin A peptides	Serum antibody titer against: peptide	ricin A	ricin
1-12	100	0	70
1-12-BSA	100	0	0
17-31	300	25,000	50
17-31-BSA	300	20,000	250
32-49	10,000	1,000	200
32-49-BSA	10,000	1,000	200
50-67	90	0	100
50-67-BSA	nd	nd	nd
84-100	10	0	100
84-100-BSA	1,000	300	100
102-118	0	0	100
102-118-BSA	0	100	40
119-136	30	0	0
119-136-BSA	0	0	0
137-152	0	0	0
137-152-BSA	500	100	0
154-166	500	0	100
154-166-BSA	500	100	0
170-178	0	0	100
170-178-BSA	0	0	200
175-191	500	0	40
175-191-BSA	10	1,000	10
178-188	0	0	50
178-188-BSA	0	0	200
186-196	100	50	100
186-196-BSA	700	500	350
192-207	10	0	200
192-207-BSA	nd	nd	nd
208-226	0	0	0
208-226-BSA	nd	nd	nd
221-233	50	0	0
221-233-BSA	100	0	0
227-243	20	0	0
227-243-BSA	600	0	100
230-240	0	0	0
230-240-BSA	50	0	0
238-249	60	0	0
238-249-BSA	80	0	10
254-265	70	0	70
254-265-KLH	1,000	0	30
ricin A	N/A	500,000	500,000
ricin B	N/A	500	100,000
ricin toxoid	N/A	500,000	500,000

Serum from mice immunized with peptides or peptide-protein conjugates were assayed for antibody titers by ELISA. Results are presented as titer, which is expressed as the recipocal anti-peptide serum dilution that yields an optical density that is equal to the optical density of normal mouse serum plus 2 standard deviation units.

Table 5

BINDING OF SERUM FROM MICE IMMUNIZED WITH
RICIN B PEPTIDES TO THE IMMUNOGEN, RICIN B, AND RICIN

	Serum antibody titer against:		
Ricin B peptides	peptide	ricin B	ricin
20-33	0	0	500
20-33-KLH	90	0	0
40-57	0	0	0
40-57-BSA	500	0	0
86-101	0	0	0
86-101-BSA	80	0	0
163-174	0	0	0
163-174-BSA	0	0	0
175-187	0	0	0
175-187-BSA	2000	0	0
188-201	100	0	0
188-201-BSA	0	0	0
206-222	0	0	0
206-222-BSA	100	0	0
245-255	0	0	0
245-255-BSA	50	50	0
ricin A	N/A	1,000	500,000
ricin B	N/A	100,000	100,000
ricin toxoid	N/A	250,000	500,000

Serum from mice immunized with peptides or peptide-protein conjugates were assayed for antibody titers by ELISA. Results are presented as titer, which is expressed as the recipocal anti-peptide serum dilution that yields an optical density that is equal to the optical density of normal mouse serum plus 2 standard deviation units.

84-100-BSA, 102-118-BSA, and 175-191 were challenged with 350 ng of ricin injected ip. Survival rates were 90%, 80%, 78%, 90%, and 60%, respectively. In the previous experiment, peptide 175-191 had produced 100% survival rate, mice being challenged with 300 ng of ricin given ip. In another challenge experiment, mice immunized

with peptides 32-49, 32-49-BSA, 84-100-BSA, and 102-118-BSA were challenged with 350 ng of ricin given ip. Survival rates were 60%, 60%, 75%, and 70%, respectively. In these studies we found that control animals injected with PBS or BSA in Freund's adjuvant by our standard immunization schedule were not killed (10-20% mortality) by a lethal dose of ricin (350 ng/mouse), although the results of previous challenge experiments showed 89-100% lethality in control groups. A review of the records of these experiments and consultation with the laboratory technician involved in these experiments revealed that the CFA control animals were not consistently immunized with PBS in IFA at the time the other animals in the experiment were receiving booster injections of peptide in IFA.

We performed additional experiments to ascertain the reason for the lack of lethality in these animals. We repeated our initial LD_{100} determination experiment to examine whether the ricin used in the challenge experiments had deteriorated. In complete agreement with the results of our initial experiment, untreated mice injected with 350 ng of ricin died within five days of challenge. In a subsequent experiment, we compared the mortality of normal, untreated mice and mice immunized with PBS in CFA and IFA, and found that 9/10 of the normal, untreated mice were killed with 350 ng of ricin whereas only 1/10 of the PBS/Freund's treated mice died. This result was not due to a difference in effective dose (i.e., mg/kg) since the mean weights of both groups of animals were equivalent, and we have never found antibodies that bind ricin in the serum of

animals immunized with CFA and IFA. The protection from ricin intoxication seen in animals injected with PBS in CFA/IFA may be due to the extended presence of the adjuvant in the peritoneum. Our standard immunization schedule consists of a primary immunization with 100 µg peptide in CFA (0.05 ml sc at two sites, and 0.1 ml ip) on Day 0, followed by booster immunizations of 100 µg peptide in IFA (0.2 ml ip) on Days 21 and 35. Challenge with ricin (0.2-0.5 ml in PBS ip) is performed on Day 49. Examination of the mice on Day 49 showed the persistence of Freund's adjuvant in the peritoneum; this adjuvant may act as a ricin "sink" when the mice are challenged ip. Injection into or association of the ricin with the adjuvant may delay the entrance of the ricin into the tissues, which in turn may reduce the maximal systemic concentration of ricin. This reduction may allow the animals to cope with the dose of ricin and survive the challenge.

To address this problem, we performed challenge experiments in which the mice were challenged iv with a lethal dose of ricin. This route of challenge, which produces mortality at a slightly lover dose of ricin than by the ip route, allows the effective evaluation of the efficacy of the anti-peptide antibodies in providing protection; the result is not influenced by the presence of residual adjuvant in the peritoneum. We performed a second experiment to determine whether the iv LD_{100} is the same in adjuvant-treated mice as in normal mice. The results, presented in Table 6, show that the LD_{100} in adjuvant-treated mice is approximately the same as in

normal mice. Once the LD_{100} was verified, we performed studies to determine the ability of peptides to induce protection. In these experiments, mice were immunized with peptide in CFA on Day 0, then boosted with peptide in IFA on Days 21 and 35. Mice were challenged iv with 200 ng of ricin (determined as the iv LD_{100}) and the survival of the mice was monitored. Results of three experiments (Table 7) show that none of the mice immunized with the ricin peptides gave protection from a lethal dose of ricin. Although these peptides do elicit antibodies that bind to ricin, the relatively low titer of antibody (10-350, compared to 500,000 for toxoid-immunized mice) may result in the lack of protection.

Table 6

DETERMINATION OF LD_{100} OF MICE
INJECTED INTRAVENOUSLY WITH RICIN

	Percent Survival (n = 9-10)		
Dose (ng)	Expt. 1	Expt. 2	Expt. 3
800	0	nd	0
400	0	nd	0
200	0	0	0
100	100	67	100
50	100	100	nd
25	100	100	nd

In Expts. 1 and 2, normal untreated mice were injected intravenously with various doses of ricin in 0.2 ml of PBS. Survival was monitored for 30 days, and results were expressed as percent survival. In Experiment 3, mice that had been injected ip once with PBS in CFA and twice with PBS in IFA were challenged with various concentrations of ricin. nd = not determined.

Table 7

RESISTANCE OF PEPTIDE-IMMUNIZED MICE
TO A LETHAL CHALLENGE WITH RICIN

	No. survivors/no. total mice per group		
Immunogen	Expt 1	Expt 2	Expt 3
PBS	0/10	0/10	0/10
BSA	0/8	nd	0/10
ricin toxoid	8/8	10/10	10/10
A(1-12)	nd	0/10	nd
A(17-31)-BSA	nd	nd	0/10
A(32-49)	0/8	0/9	nd
A(32-49)-BSA	0/8	nd	0/9
A(50-67)	nd	0/8	nd
A(84-100)-BSA	0/8	nd	0/10
A(102-118)-BSA	0/8	nd	nd
A(154-166)	nd	nd	0/10
A(186-196)	nd	nd	0/10
A(186-196)-BSA	nd	nd	0/10
A(227-243)-BSA	nd	nd	0/10

Groups of mice were immunized with the various immunogens emulsified in CFA on Day 0. Mice received booster immunizations on Days 21 and 35, and were challenged intravenously with 200 ng ricin on Day 49. Survival was monitored for 30 days.

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